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Symbiotic Nitrogen Fixation Technology

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Principles and Practice of Field Designs to Evaluate Symbiotic Nitrogen Fixation

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I. PURPOSE OF FIELD TRIALS

The capacity of a specific plant-*Rhizobium* association to fix nitrogen under optimal conditions can be defined as "potential nitrogen fixation" as opposed to "actual nitrogen fixation" (Lie, 1981). Actual nitrogen fixation is measured in the field, where factors such as soil moisture, temperature and light may prevent the full expression of the symbiosis. Field testing is an essential step in research designed to assess nitrogen-fixing capacity of a host plant or a *Rhizobium* strain; to determine strain competitiveness or persistence; to determine the need for inoculation; and to select for superior host plants, rhizobial strains, or specific host genotype-rhizobial strain combinations.

A. Considerations in Field Experimentation

Field experiments involving nitrogen fixation generally represent a large investment of time and resources. The failure of a field experiment is, therefore, usually quite costly. Thus field studies should be planned and conducted carefully to reduce the potential for failure.

Several factors should be considered in planning a field study. The inherent variability of the soil, the nutritional level of the soil, the previous and present cropping system, the selection of treatments, the evaluation procedures, and the experimental design can affect the reliability and usefulness of the data. Brockwell et al. (1982) suggest that the characteristics of naturally occurring populations of rhizobia at the field site, an effective inoculation technique with known numbers of viable rhizobia which can be identified when reisolated from the experiment, good-quality seed, and adequate controls are also necessary. The objectives of this chapter are to provide guidelines for planning and conducting field experiments in symbiotic nitrogen fixation. It is impossible to provide all the details necessary to suit all field experiments, but important considerations are discussed. The researcher is provided with references to important literature citations for additional detail.

II. PLANNING FIELD EXPERIMENTS

A. Objectives of the Study

A clear, concise reason for conducting a study is essential to plan a field experiment. The purpose of the study will affect selection of the field site, the treatments employed, the traits evaluated, the experimental design, and the interpretation of the data. For example, the objectives of legume inoculation trials may be to answer the following questions (FAO, 1983):

Is it possible to increase traditional legume crop yields by using inoculation?

What is the effect of inoculation on the yields of introduced crops, and is it possible to grow new legumes effectively by relying solely on inoculation as a source of nitrogen?

What savings can be made by using inoculation rather than applying chemical nitrogenous fertilizer?

What is the effect of inoculation, and what savings can be made in terms of the following crop in the rotational system?

Is one inoculum more efficient than another for a given region?

If the objectives are complex, several experiments may be required to answer all the desired questions. For example, the international legume inoculation trials originally proposed by NifTAL consist of three experiments (Harris, 1979). The best available rhizobial strains for a particular crop species are tested in an initial experiment designated experiment A. Since the strains used for experiment A have not been selected for the local cultivar or for the local agroclimatic conditions, a lack of response to inoculation does not necessarily mean that inoculation is of no value. A second experiment is conducted in pot culture using a local rhizobial strain(s) adapted to the legume(s) being tested under the specific agroclimatic conditions. The third experiment is conducted in the field with a *Rhizobium* strain or mixture of strains selected for the local cultivar under its own growing conditions.

B. Selection of a Field Site

The selection of a field site for symbiotic nitrogen fixation research should be influenced by practical considerations. It may be chosen because it represents an area or possible area for legume crop production which requires investigations, or it may be a site chosen because it meets the requirements of the prescribed experimental conditions. For example, an experimental site for *Rhizobium* strain testing may be selected because it has a low level of soil nitrogen with few or no naturally occurring rhizobia, whereas a site designed to test strain competitiveness and persistence may be chosen because it has an established population of appropriate rhizobia.

Regardless of whether the field site is chosen for optimum experimental conditions or because it is representative of the production area, the field site should be nearly level with a homogeneous, uniform soil type since soil heterogeneity due to differences in soil topography, soil moisture, soil fertility, and previous crop is a source of considerable variability in field experiments (LeClerg et al., 1962).

Selection of Site for Optimum Conditions

The optimum field site will vary with the objectives of the experiment. For an inoculation response test, or one to compare rhizobial strains,

or a test to evaluate specific host-strain combinations, an ideal site should have a low level of soil nitrogen, no drainage problems, no previous legume crop, no particular soil salinity or acidity problem, and have no or a limited population of *Rhizobium*, usually less than 1000 per gram of soil (NifTAL) (Harris, 1979; Weaver and Frederick, 1972). Sites for experiments to evaluate strain competitiveness and/or persistence and to identify host plants with superior nitrogen-fixing ability in symbioses with indigenous rhizobia should have similar characteristics except that the site should have an established population of desirable rhizobia.

Selection of Representative Site

If the experiment is designed to demonstrate the effect of symbiotic nitrogen-fixation treatments or to select genotypes for an already established cropping system, the site selected should be as representative of the area or cropping system as possible. Not only the soil characteristics but the growing season, and whether the legume will be sole- or intercropped, should also be considered for experiments in a tropical environment with multiple growing seasons.

C. Analysis of Soil Fertility

Several soil characteristics, including the status of soil nutrients, have been identified that affect survival of rhizobia or efficacy of a resulting symbiosis. Soil pH, nitrogen, and phosphorus often have large effects on growth, survival, and invasion of rhizobia (Munns, 1978; Robson, 1978). At low soil pH, aluminum and manganese are often present at levels that are phytotoxic to certain plants and rhizobial strains. The lack of phosphorus may limit legume growth, particularly in tropical soils. The effect of nitrogen, mineralized in the soil or applied as fertilizer, depends on the level. A low-level application is often recommended to stimulate seedling growth prior to effective nodulation, while higher levels inhibit nodulation and subsequent nitrogen fixation. In addition, calcium, boron, magnesium, sulfur, and molybdenum are required for efficient nitrogen fixation, while copper, zinc, and cobalt may also be involved (Robson, 1978). The extent to which any or all of these nutrients affect the symbiotic relationship is usually site specific and must be considered when evaluating the effectiveness of symbiotic nitrogen fixation. Because the soil nutrient status often limits nitrogen fixation, a soil analysis should be made to determine the fertility requirements for each site. NifTAL (FAO, 1983) recommends that each analysis include the following in priority order:

1. Free nitrogen, available phosphorus, exchangeable potassium, calcium, and magnesium
2. Exchangeable aluminum and manganese if the respective pH is lower than 5.2 and 5.6

3. pH and level of organic matter
4. Available proportions of certain mineral elements if deficiencies are suspected

A technical handbook prepared for FAO (1983) recommends that a more detailed preliminary soil analysis be completed before conducting a field trial on symbiotic nitrogen fixation. The following analyses were suggested: soil particle size (clay + silt); soil acidity in pH (colorimetric and potentiometric method-glass electrode); exchangeable bases (calcium, magnesium, potassium, and sodium); if the pH is lower than 5.2 and 5.6, respectively--exchangeable aluminum and manganese; total phosphorus; available phosphorus; total nitrogen; NH_3 and NO_2; organic carbon; if absence is suspected--cobalt and boron; if absence or excess is suspected--molybdenum.

Although the latter analysis is more complete, it is often difficult to obtain even the simpler analysis recommended by NifTAL. In that case, the treatments should be planned to determine whether fertility or nutrient status of the soil is limiting nitrogen fixation. In most studies of symbiotic nitrogen fixation, the fertility level for the legume crop should be the optimal level for the crop species under test since the plant must live and grow before the symbiotic process can be evaluated.

D. Selection of Treatments

Singularly or in Combination

Experiments can be simple or factorial based on the number of factors or variables to be studied simultaneously. The simple experiment is often used because of its simplicity. For the simple experiment, one variable, such as the effect of rhizobial strain inoculation, is studied separately. However, information from a simple experiment is of limited usefulness because inferences cannot be made about potential interaction between factors such as inoculation with rhizobial strains onto different host cultivars or their response to different fertilizer treatments.

Two or more simple experiments can be combined into a single factorial experiment so that the response of the different factors singularly as well as different interactions can be determined. The factorial experiment has greater efficiency and greater comprehensiveness than simple experiments.

Response to Inoculation Trials

Knowledge of nodulation specificity and effectiveness of symbioses between tropical legumes and *Rhizobium* has increased substantially. The continued study of both familiar and poorly understood genera,

in a wide range of soil and climatic conditions, has demonstrated meaningful interaction between host and strain as well as favorable responses to inoculation with effective strains in the field. These studies contradict a popular consensus that many tropical legumes nodulate effectively with indigenous strains and consequently do not respond to inoculation.

Simple inoculation trials which are most often simple experiments, in that only inoculation is considered as the treatment factor, are essential prerequisites for introduction of legumes into new areas and the selection of appropriate strains of *Rhizobium* for production (Nutman, 1976; Date, 1982). Treatments in inoculation trials consist of uninoculated control plants and inoculated and inoculated-plus-nitrogen plants. The uninoculated control plants indicate the presence or absence of indigenous strains and if indigenous strains are present, provide some assessment of their symbiotic effectiveness compared to plants inoculated with selected rhizobial strains. The inoculated treatment provides a comparison of the ability of test strains to colonize the host root environment and to compete for nodule sites. An inoculated-plus-nitrogen treatment should be included to ensure that the legume plant has the potential to grow well once nitrogen is provided and that growth is not limited by some other deficiency.

Results from this set of treatments can be interpreted as follows (Date, 1982):

1. No nodulation, poor plant growth, and severe nitrogen deficiency of uninoculated, inoculated, and inoculated-plus-nitrogen control plants indicate an absence of suitable native rhizobia and factor(s) other than nitrogen are limiting plant growth.
2. Absence of nodules and poor growth of uninoculated and inoculated plants but healthy growth of plants receiving added nitrogen indicates an unsatisfactory inoculum strain or soil conditions inadequate for growth and infection of rhizobia.
3. Absence of nodules and poor growth of uninoculated plants, but effective nodulation and growth of inoculated plants similar to the nitrogen treatment, indicates successful nodulation and N_2 fixation by inoculated strain(s).
4. No nodulation and poor growth of uninoculated plants along with nodulation but poor growth of inoculated plants relative to the nitrogen treatment indicates that the inoculum strain is infective but ineffective for N_2 fixation.
5. Nodulation but poor growth of uninoculated treatment indicates the presence of unsuitable native rhizobia. Inoculated plants effectively nodulated and well grown indicates that inoculum strain(s) is both competitive and effective.
6. Both uninoculated and inoculated plants well nodulated but where growth is poor relative to nitrogen treatment indicates

that (a) native rhizobia are ineffective but compete with introduced strain, or (b) both introduced and native rhizobia are ineffective.

7. All treatments grow well; no response. Nodules on inoculated plant treatment may be native or introduced. Tests to identify nodule occupancy are needed.

Information obtained from rhizobial assessment trials with the three sets of treatments as just described will aid the researcher in determining criteria for assessing suitable strains of *Rhizobium*. For example, when the uninoculated plants have no nodules and poor growth but the inoculated plants are nodulated and have growth similar to the nitrogen treatment, the criterion for strain selection is effectiveness for nitrogen fixation. However, when both uninoculated and inoculated plants are nodulated but growth is poor, selection for competitive ability is essential. When all treatments grow well, inoculation is not essential.

Inoculation response trials can be factorial and include additional treatments. For example, an experiment can consist of three replications of each inoculant treatment with main plots split for phosphorus application.

For perennial species the persistence of a strain is an important criterion for selecting suitable strains. Experiments must be maintained and sampled over several years. For annual legumes plots can be resown each year with uninoculated seed.

Selection of Improved Host Plants

The genotype of the host plant can affect N_2 fixation through direct effects on symbiosis and indirectly through the growth habit and plant vigor. The direct effect of host genotype on symbiosis is generally described in terms of nodulation effectiveness and selectivity of the host for certain rhizobia from a mixed population (Vincent, 1984). The growth habit and plant vigor affects N_2 fixation indirectly through influence of the size of the root system, time to flowering and seed set, and tolerance to edaphic factors limiting plant growth and development. In addition to choosing improved rhizobia, selection of superior host plants is essential to ensure high levels of fixation.

Improved grain legumes, forage, and pasture legumes should be adapted to the production area, contain disease, and pest resistance and produce satisfactory yield of high quality grain, forage, or pasture. Considerable variation for nodulation and N_2 fixation has been observed in most legumes. However, few breeding programs have exploited this variability to develop improved cultivars with increased N_2-fixation potential.

The breeding methods used to develop improved host plants vary depending on the reproductive system of the plant. The crop plants

within the Leguminosae include both self- and cross-pollinated species as well as those that are predominantly one or the other (Table 1). For self-pollinated crops, use of breeding methods that allow the development of segregating populations of lines (families) such as the modified pedigree method (MPM) (Brim, 1966) or the inbred backcross line method (IBLM) (Bliss, 1981) offer several advantages. The IBLM has been used successfully for increasing fixation in common bean (McPerson et al., 1981; Bliss et al., 1985). A population of nearly homozygous lines was developed through repeated backcrosses to an adapted parent after an initial cross to a high-fixing donor parent. After the last backcross two to three generations of selfing by single-seed descent produced the population of families. The resulting inbred backcross lines are similar to the recurrent parent for most traits, but differ for the traits of the donor parent. Selection for differences in fixation are made at this time. Because the seeds of each family are not limited, they can be replicated several times at different locations to increase precision and the selected families used for further crossing, additional studies, or released as potential new cultivars. Original parents and adapted local cultivars should be included for comparisons.

Table 1 Genera, Major Crops, and Type of Reproduction of Family Leguminosae

Genus	Crop	Reproduction
Arachis	Peanut	Self-pollinated
Cajanus	Pigeon pea	Usually self-pollinated
Cicer	Chickpea	Self-pollinated
Glycine	Soybean	Self-pollinated
Lens	Lentil	Self-pollinated
Medicago	Alfalfa	Cross-pollinated
Phaseolus	Common bean	Self-pollinated
	Runner bean	Self-pollinated
	Tepary bean	Usually self-pollinated
	Lima bean	Self-pollinated
Pisum	Pea	Self-pollinated
Vicia	Bean	Cross-pollinated
Vigna	Cowpea	Self-pollinated

Breeding procedures for increasing nitrogen fixation of cross-pollinated species such as alfalfa may be more complicated. Phenotypic recurrent selection for increased N_2 fixation in the greenhouse was ineffective when the populations were evaluated in the field (Barnes et al., 1981; Heichel et al., 1981). A breeding procedure where the progenies of individual clones are evaluated in the field will probably be required to increase nitrogen fixation. The progenies could be evaluated similarly to the inbred lines described for self-pollinated species.

Host-Strain Specificity

Field studies designed to estimate host-strain specificity are an extension of an experiment for determining the response to inoculation. Treatments consist of several host genotypes inoculated with different rhizobial strains. An uninoculated check and a nitrogen treatment for each genotype should be included. Generally, the two factors (hosts and rhizobial strains) are evaluated in all combinations. This may increase the number of treatments substantially requiring the experiment to be planted in a split-plot design. Generally, rhizobial strains should be assigned to main plots and host genotypes to subplots because the application of inoculum to whole areas within a block will reduce the risk of contamination.

Other Studies

Field studies to estimate the amount of nitrogen fixed by legumes are often conducted. These studies and the methods useful in determining nitrogen fixation of legumes are described by LaRue and Patterson (1981) and elsewhere in this text.

E. Design of a Field Experiment

Experimental design involves planning a field experiment to permit the collection of unbiased data with maximum accuracy and precision at a reasonable cost. The planning stage includes consideration of soil heterogeneity, nature and importance of experimental error, principles of field experimentation, field plot technique, and appropriate experimental designs (LeClerg, 1966).

The greatest source of experimental error in field experiments is generally due to soil heterogeneity (LeClerg et al., 1962). Since soil heterogeneity has long been recognized as a source of experimental error, experimental designs have been developed to eliminate, or at least minimize, the effect of soil heterogeneity. The general practice for controlling soil heterogeneity in field experiments is to decrease the size of the plot and increase the number of replications. The repetitions of a plot are then scattered over the test area in order to

sample different soil conditions. Although efficient experimental designs can remove a portion of the variability due to soil conditions, careful selection of the experimental site is also important in studies of symbiotic nitrogen fixation.

Variability in field experiments comes from two principal sources: (a) inherent variability which exists in the experimental material, and (b) variability that results from lack of uniformity in the physical conduct of an experiment (Steel and Torrie, 1960). The failure of plots treated alike to perform similarly is experimental error (Fisher, 1951). The degree of confidence associated with field data depends on the magnitude of experimental error. Experimental error should be reduced as much as possible by reducing the inherent variability of the experimental material and using careful experimental techniques. A good experimental design simply estimates the magnitude of the remaining uncontrolled experimental error.

Principles of Field Experimentation

Field experiments are designed and performed carefully so that appropriate inferences can be made from the data. To make inferences as precise as possible, replication and randomization of the experiment are practiced. Since the soil of an experimental site is assumed to be variable, treatments must be replicated. Replication provides an estimate of experimental error and reduces the uncertainty of the data. Multiple observations from a plot do not represent true replication but are subsamples and allow estimation of sampling error.

Because soil heterogeneity is universal in field sites, treatments cannot be compared on an identical soil at the same time but must be compared on different plots of an experimental site. This procedure is satisfactory only if treatments are assigned at random to the plots of the site.

Certain restrictions on the random arrangement of treatments in a replicated experiment can be imposed such that some of the total variability not relevant to treatment comparisons may be eliminated. This is called local control and is synonymous with proper experimental design.

Experimental Designs

Each field study presents conditions that must be considered in the choice of an appropriate experimental design. However, the simplest design that fulfills the requirements of the experiment should be used (LeClerg et al., 1962). The randomized complete block design can be utilized for most nitrogen-fixation experiments. A Latin square design can be used to remove variability from soil heterogeneity in two directions. It is very efficient but it is usually not practiced with more than eight treatments (Little and Hills, 1978). A randomized complete

block design is usually effective for experiments having up to 15 treatments. For greater numbers of treatments in a single experiment, incomplete block designs should be considered. When it is convenient to arrange the plots of a multiple-factor experiment so that all factors are not distributed over the replication and the factors are not of equal importance, a split-plot design can be used. The split-plot design provides a more precise comparison of treatments assigned to subplots and a less precise comparison of the whole-plot treatments.

Most inoculation trials such as the INLIT trials conducted by NifTAL (Harris, 1979) or the FAO legume inoculation trials (FAO, 1983) specify a randomized complete block design, although Date (1976) suggests that split plots might be useful in inoculation response trials where a trial is extended to include additional treatments, such as the presence or absence of phosphorus.

A statistician should be consulted on the proper experimental design and data analysis if the treatments are numerous or if the experiment is not one of the routine symbiotic nitrogen-fixation field studies.

III. PLANTING A FIELD EXPERIMENT

A. Preparation of Experimental Site

The entire experimental area, including borders, should be treated uniformly. Cultural practices such as plowing, disking, harrowing, and any basic fertilizer treatment used over the entire test should be done before planting using procedures common to the region. Seedbed preparation should be appropriate for planting the legume crop chosen for the experiment.

B. Plot Layout

The specific layout of a trial designed to determine the response to inoculation should vary according to the specific objectives. An example of a standardized experimental method for testing the value of inoculation has been developed by NifTAL (Harris, 1979). NifTAL initiates an experiment with rhizobial strains considered to be the best available for the test legume. Since the rhizobial strains are not selected initially for the local cultivar or for the local agroclimatic conditions, negative results do not necessarily constitute a definitive evaluation. Thus NifTAL recommends a three-phase trial as follows:

1. Experiment A, based on the best available rhizobial strains and local cultivar of the host species.
2. Experiment B, a pot experiment using site soil which compares local strains with strains tested in experiment A to select a strain adapted to the legume being tested under specific agroclimatic conditions.

3. Experiment C, conducted with a *Rhizobium* strain (or mixture) specifically selected for the local cultivar grown under local agroclimatic conditions. The plot layout and arrangement of plots for experiment C are shown in Figure 1.

The experiment is laid out in a randomized complete block design with four replicates. Rows are 7.5 m long with four rows per plot. The width of the plot is from 2 to 4 m, depending on the legume species being evaluated. The plots are designed for early season sampling of nitrogen fixation and a final harvest of yield (Figure 2).

C. Methods of Inoculating Field Studies

Types of Inoculant

Numerous methods of inoculating legumes have been employed depending on the type of inoculant available. Most commercial inoculants are based on solid carriers, mainly soil or peat. The survival of rhizobia has generally been better when solid-based inoculants are applied to the seed than when other forms of inoculant are used. However, several other types of inoculant, such as agar cultures, liquid cultures, or lyophilized cultures, have been used experimentally.

Application of Inoculant

Principles of inoculation for field experiments: Brockwell (1982) lists some general principles of legume inoculation that apply to field experimentation regardless of the method of inoculation. They include:

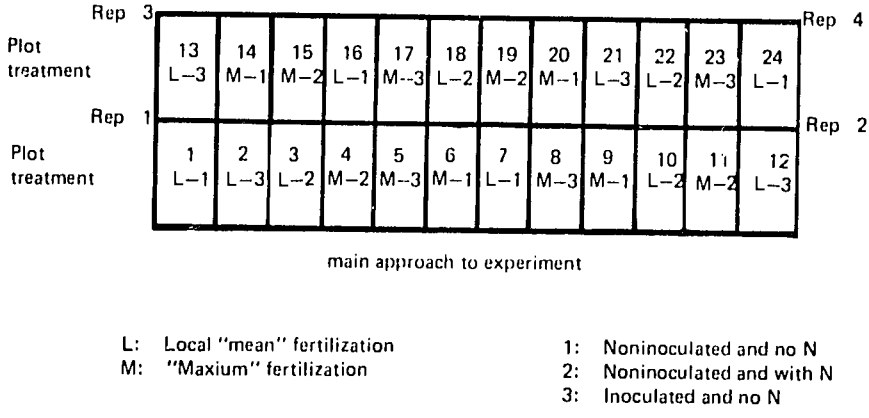

Figure 1 Trial plot layout.

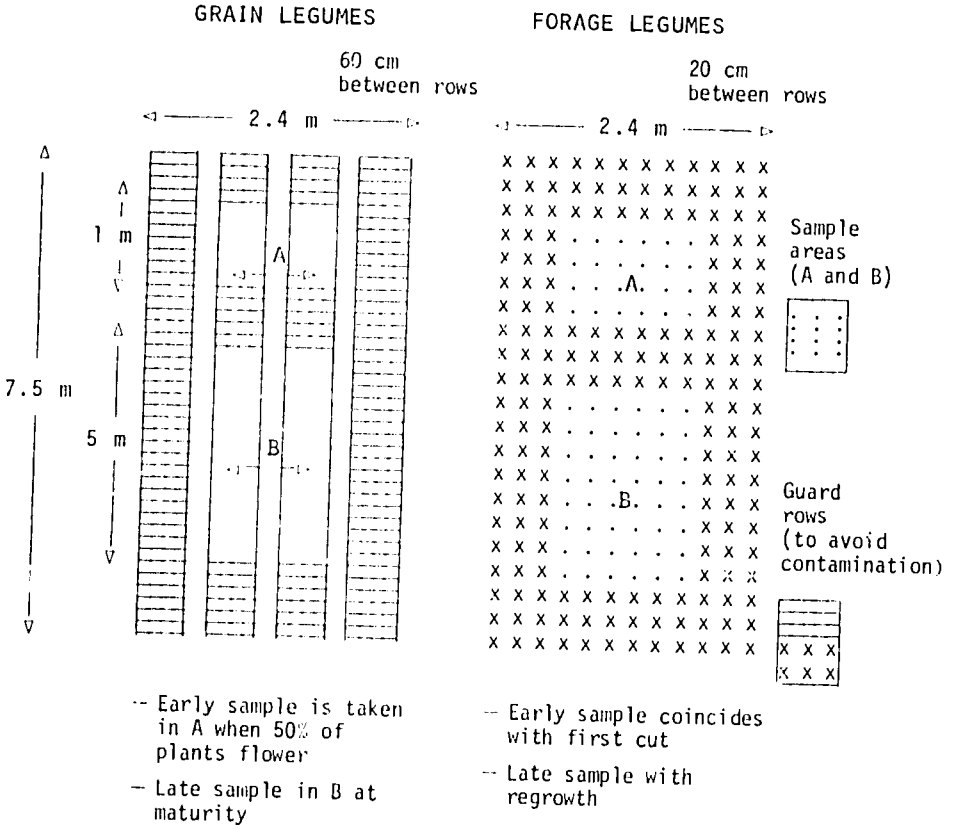

Figure 2 Plot dimensions and sampling area.

1. When uninoculated controls are included, they should be handled before inoculated treatments to reduce the risk of contamination of the controls.
2. The number of inoculant rhizobia should be as high as feasible depending on the objective of the experiment since the presence of large numbers of inoculant rhizobia reduces contamination by other rhizobia.
3. Designing and managing a field experiment with the awareness that rhizobia are mobile organisms and can be moved easily from plot to plot.
4. Rhizobia are incompatible with many pesticides used as seed treatments.

Methods of application

1. *Seed inoculation.* Inoculants can be applied to the seed before planting or they can be applied directly into the seedbed. The most widely used methods of inoculating seeds are slurry inoculation and seed pelleting, while solid carrier-based inoculants and liquid inoculations are usually applied into the seedbed.

   a. *Slurry inoculation.* The rhizobial culture is applied to the seed as a water suspension or the inoculant is mixed with moistened seed. The seed must be dried before planting, thus killing a proportion of the rhizobia. More inoculant can be attached to the seed by using an adhesive such as a 10% solution of sugars or gum arabic in the slurry.

   b. *Seed pelleting.* Legume seeds may be coated with lime to promote nodulation. Advantages include protection of the rhizobia against toxic substances contained in some legume seed coats, unfavorable physical and chemical conditions in the soil, competition from soil microflora, and the effects of acid fertilizers (Brockwell, 1977). Brockwell (1982) described one method for pelleting seeds using standard laboratory equipment that can be used for small lots of seeds. Materials used for seed coating include calcium carbonate, dolomitic limestone, and gypsum.

2. *Seedbed inoculation.* In several situations the application of rhizobia to seed is an inefficient method of inoculation (Burton, 1974; Brockwell et al., 1982). An alternative method of inoculation such as application of the rhizobia to the soil can be used. Inoculant can be applied to the seedbed as a solid inoculant or as a liquid.

   a. *Solid inoculant.* A solid inoculant or soil implant inoculant is produced by coating a solid granulated material with peat inoculant in an adhesive. A suitable adhesive is a 25% aqueous solution of gum arabic without preservatives. The peat inoculant is thoroughly stirred into the adhesive. The resulting suspension is poured onto a granulated material, which can be any inert, particulate free-flowing material, and mixed until the material is evenly coated. For large experiments solid inoculant can be applied through a fertilizer attachment or pesticide applicator of the planter. For smaller experiments it can be applied by hand to the row along with the seed. Solid inoculant is especially suited for experiments on the rate of inoculant or for the inoculation of numerous small samples.

   b. *Liquid inoculation.* Excellent nodulation can be obtained by applying a liquid inoculum into the row beside or beneath the seed. A peat culture of rhizobia from frozen or concen-

trated broth culture is mixed with water and can be applied by a gravity-flow applicator or sprayer. The method works well with small experiments but can create problems with blocked nozzles when used with spray equipment. Brockwell (1982) appraised several inoculation methods suitable for field experimentation (Table 2).

IV. EVALUATION OF TREATMENTS

A. Data Collection

Appropriate measures of the response to inoculation are plant dry matter, total plant nitrogen accumulation, and seed yield for grain legumes and dry matter yield for forage legumes. However, there are many traits associated with nitrogen fixation that can be described during plant growth in the course of an experiment. These include earliness to nodulation, nodule number, nodule mass, nodule color, distribution of the nodule population, longevity of the nodule population, and visual nodulation scores.

It is common for legumes to show a visual response to inoculation that does not necessarily correspond directly to seed yield increase. Thus it is desirable to obtain some measure of the early response to a treatment to distinguish between no response and a potential for response.

B. Measurement of Traits

Nodulation is generally assessed from 6 to 20 weeks after planting, depending on the legume and the seasonal conditions, by sampling a number of plants in each treatment. Nodule number is a valuable criterion in the early stages of plant development, but it is of little use thereafter. At that point the best criterion is nodule dry mass or nodule dry mass per unit dry weight of the whole plant or root system. In experiments where plants with different growth rates are being compared, it is important that measurements be made at similar stages of maturity (e.g., the R3 stage) rather than at the same time after emergence (McFerson et al., 1982). After determining the nodule mass one should determine whether these nodules are effective. As a general rule, effectiveness can be gauged by the degree of pink or red coloration of the nitrogen-fixing bacteroid tissue inside the nodule—white or green being inactive. Another measure of effectiveness is use of acetylene reduction methods (Hardy and Holsten, 1977; Masterson and Murphy, 1976, 1980; Ham, 1977). The acetylene reduction assay has the advantage of sensitivity, speed, and economy (LaRue and Patterson, 1981), although it has been criticized by some as being of questionable value (Minchin et al., 1983; Sinclair et al., 1976). Because

Table 2 Methods and Appraisal of Legume Inoculation

Method	Procedure	Appraisal
Dusting	Dry, peat-based inoculant poured on seed in planter	Quick, simple; ineffective since much of the inoculant is lost before the seed reaches the soil, thus no longer recommended
Slurry	Peat-based inoculant suspended in water and applied to seed, mixed, and allowed to dry before planting	No special equipment required; necessary to dry seed away from direct sunlight; some splitting of seed may occur during handling; slow and labor consuming; reinoculation necessary if not planted within 48 hr; not suitable when fungicides or insecticides applied to seed
Solid inoculant	Polyethylene beads, marble chips, or other freely flowing material coated with peat-based inoculant or peat-based inoculant extruded into freely flowing pellets and applied in the row with seed	Gives excellent nodulation; maintain viability well; suitable for inoculation of pesticide-treated seed
Seed pelleting	Peat-based inoculant suspended in adhesive, applied to seed, mixed together, then finely ground coating material added to sticky seed and mixed until each seed is evenly coated	Protects inoculants against deleterious soil environmental factors; time-consuming preparation; coating material may come off and cause blockage in planters; high inoculant mortality rate observed in many pelleted seed samples
Spray inoculation	Peat-based inoculant suspended in water and sprayed into seedbed alongside seed	Gives excellent nodulation; no storage problems provided that peat inoculant kept cool until suspended in water; suitable for inoculation of pesticide-treated seed; no complicated preparation procedure; easy to apply; blocked delivery nozzles can be a problem
Pre-inoculation	Inoculation of seed with rhizobia prior to sale	Convenient for farmers; mortality rate of inoculant often high, resulting in low levels of inoculation

the plant is usually destroyed during the assay, acetylene reduction is a single-point, indirect estimate of nitrogen-fixation potential. Thus an estimate of fixation by a crop or the identification of superior treatments requires a summation of several assays over a growing season (Arrendell et al., 1985), or other more accurate methods (e.g., ^{15}N isotope application).

The effect of treatments can also be measured by total plant nitrogen or the nitrogen content of any plant part. The final evaluation of a treatment effect is to measure economic yield at harvest (i.e., grain yield or forage yield).

C. Analysis and Interpretation of Data

No study is complete until the data have been summarized, analyzed, and interpreted properly. An analysis of variance or regression analysis providing a test of significance of treatment means is the minimum analysis required. Additional analysis such as covariance or trend analysis may be needed to interpret the results.

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